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TITLE: A Potential Therapeutic Role of J Series Prostaglandins  
in PPAR $\gamma$  Mediated Treatment of Breast Cancer

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## **Introduction**

The peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), is a potential therapeutic target for the treatment of breast cancer but the endogenous ligand for PPAR $\gamma$  is not yet known. Recent data suggest that the endogenous ligand of PPAR $\gamma$  may be a bioactive metabolite of arachidonic acid that is synthesized in normal breast tissue. Activation of PPAR $\gamma$  with different agonists (e.g. 15deoxy $\Delta$ 12,14PGJ<sub>2</sub>, troglitazone) elicits different physiological responses in breast cancer cells (i.e. differentiation or apoptosis) raising questions of the role PPAR $\gamma$  plays in normal breast cell physiology. Results from our initial experiments show that prostaglandin metabolites of arachidonic acid inhibit cell cycle progression of MDA-MB-231 breast cancer cells. This cell cycle block induces apoptosis of breast cancer cells and inhibits tumor formation in nude mice. We hypothesize that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPAR $\gamma$  mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPAR $\gamma$  agonists. Understanding the metabolism of fatty acids in breast cancer cells, and elucidating the molecular and signal transduction events that are mediated by PPAR $\gamma$  agonists may lead to novel strategies for the prevention and treatment of breast cancer.

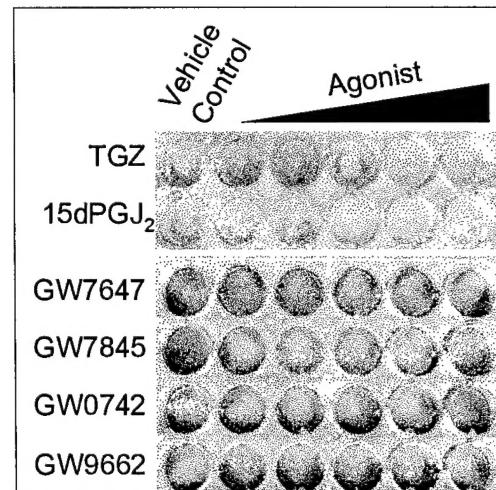
## Body

There is extensive literature on the use of retinoic acid and its derivatives, acting through their receptors (RAR and RXR), to arrest or reverse cancer in both animals and humans. Another member of the nuclear receptor superfamily, peroxisome proliferator activated receptor-gamma (PPAR $\gamma$ ), has an important role in fat metabolism and adipocyte differentiation. Although its natural ligand is not yet known, synthetic thiazolidinediones, certain fatty acids and metabolites of arachidonic acid, activate PPAR $\gamma$ . Recent data reveal that PPAR $\gamma$  is expressed in colonic tumors and metastatic breast adenocarcinomas, which raises the critical question of its functional significance in human cancers. RXR $\alpha$  and PPAR $\gamma$  agonists together have been shown to induce apoptosis of estrogen receptor positive breast cancer cell lines *in vitro* and attenuate tumor growth in mice. Our studies show that prostaglandin agonists of PPAR $\gamma$  alone inhibit cell cycle progression of both estrogen receptor positive and negative breast cancer cell lines via apoptosis and inhibit tumor formation in nude mice.

There are three specific aims for the pre-doctoral research hypothesis that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPAR $\gamma$  mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPAR $\gamma$  agonists.

- 1) The first aim is to determine the physiologic activities of different PPAR $\gamma$  agonists on the proliferation of human breast cancer cell lines and primary human breast cancer cells. We will extend our published findings to include other natural prostanoid and eicosanoid agonists (e.g. PGE<sub>2</sub>, DHA), synthetic PPAR $\gamma$  agonists (e.g. BRL49653, ciglitazone) and co-activators that can potentiate the effects of PPAR $\gamma$  agonists (e.g. 9-*cis*-retinoic acid, all-*trans*-retinoic acid).
- 2) The second aim is to determine the molecular mechanisms and signal transduction events that underlie PPAR $\gamma$  mediated differentiation or apoptosis in breast cancer cells.
- 3) The third aim is to determine the metabolism of J-series prostaglandins in normal breast tissue and breast cancer cells.

Aim 1: Our studies of other natural and synthetic PPAR $\gamma$  agonists show that several arachidonic acid (AA) metabolites, including 5- and 15-HETEs and 5-and 15-oxo-EETs, are activators of PPAR $\gamma$ . However, of all the naturally occurring metabolites tested, the terminal derivative of prostaglandin D<sub>2</sub> metabolism, 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> (15dPGJ<sub>2</sub>), remains the most potent. A major accomplishment of Mr. Clay's was his observation that the published literature cites different physiologic outcomes in various cancer cell lines according to the concentration of PPAR $\gamma$  agonist used. To this end, Mr. Clay authored a review article that documented the differing biological effects of PPAR $\gamma$  activation in diverse cell types (1). Furthermore, Mr. Clay undertook the responsibility of determining if these diverse and opposing biologic outcomes occur in a single cell type (2). In addition, after attending the PPARs Keystone Symposium in February 2000, Mr. Clay was successful in obtaining chemically synthesized selective agonists for each of the PPAR isoforms ( $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ ) from GlaxoSmithKlein (GSK). These compounds are 10,000 fold more selective for their respective receptor than for other nuclear receptors. Mr. Clay has shown that selective activation or inhibition of PPAR $\gamma$ , using the synthetic agonist GW7845 or antagonist GW9662, does not alter cellular proliferation in breast cancer cell lines (Figure 1).



Aim 2: The molecular mechanisms and signal transduction events that underlie PPAR $\gamma$  mediated differentiation or apoptosis in breast cancer cells are complex and not well understood. Mr. Clay has achieved great milestones in elucidating parts of these pathways. In a screen of 1,176 gene products by cDNA array analysis, Mr. Clay identified particular gene products that are increased in breast cancer cell lines after treatment with 15dPGJ<sub>2</sub>. Of these, the expression of the cyclin dependent kinase inhibitors p21<sup>Waf1/Cip1</sup> (p21) and p27<sup>Kip1</sup> (p27) and the cyclins D and E is increased >2 fold. Additionally, the expression of several genes involved in DNA maintenance and repair is decreased >2 fold. Mr. Clay has performed *post hoc* analysis of p21 and p27 expression by Western blot analysis to

confirm the results from the cDNA array (Figure 2) and will establish cell lines that express a dominant negative form of p21. Additionally, Mr. Clay has followed up on published reports of the effects of 15dPGJ<sub>2</sub> in other cell systems to devise a potential mechanism by which 15dPGJ<sub>2</sub>, or other cyclopentenone prostaglandins, may exert such potent anti-neoplastic activity in a variety of cancer cell types (Appendix 1). These studies have resulted in a manuscript which has been published in *The Journal of Biological Chemistry* (3). Mr. Clay will continue this line of investigation to include other gene products and further elucidate the mechanisms described. Furthermore, Mr. Clay has established breast cancer cell lines that express a dominant negative form of PPAR $\gamma$ . He has shown that transcriptional activation of PPAR $\gamma$  by 15dPGJ<sub>2</sub> is blocked in these cells (Figure 3). More recently Mr. Monjazeb, in conjunction with Mr. Clay, has utilized these dominant negative forms of PPAR $\gamma$  as well as pharmacologic inhibitors of PPAR $\gamma$  to demonstrate that the apoptotic potential of 15dPGJ<sub>2</sub> is independent of PPAR $\gamma$  activation in cancer cell lines. These results have been published in the *Journal of Lipid Research*. Furthermore, our results, as well as many recent published reports, suggest that the anti-neoplastic effects of 15dPGJ<sub>2</sub> may be linked to AA metabolism in general as it has been demonstrated that other products of AA oxidation also have potent effects on cancer cell growth and viability. These results are outlined in a peer review published in *Prostaglandins Leukot Essent Fatty Acid*.

Aim 3: Given the findings described in Aim 2, we chose to expand Aim 3 to investigating the role of AA and its metabolites in neoplastic cells rather than focusing on the metabolism of 15dPGJ<sub>2</sub> alone. We examined the anti-tumor effects of three distinct AA metabolic enzyme inhibitors Triacsin C, PLT 98625, and NS-398, which inhibit Fatty Acid Coenzyme-A Ligase 4 (FACL-4), Coenzyme-A Independent Transacylase (CoA-IT), and Cyclooxygenase (COX), respectively. We found that inhibition of AA metabolism had potent anti-neoplastic effects in a number of cancer lines including the MDA-MB-231 cell line and induced caspase-3 activation and apoptosis. We also found that these inhibitors potently increase intracellular accumulation of AA and that apoptosis was intimately linked with this apoptosis. Our results suggest that inhibitors of AA metabolic enzymes induce cancer cell apoptosis by increasing levels of intracellular unesterified AA and indicate that combination treatment strategies utilizing these inhibitors may represent a novel approach to blocking cancer cell growth. These results are included in a manuscript under preparation for submission to the *Journal of Lipid Research*.

We performed further studies to examine downstream pathway(s) whereby high cellular burdens of unesterified AA promote apoptosis. We found that many previously hypothesized pathways, including the conversion of accumulated AA to cytotoxic products or AA induced ceramide generation, are unlikely to play a key role in the apoptosis induced by AA metabolic enzyme inhibitors; however, further study is required. Given the ability of AA and its metabolites to augment gene transcription we hypothesized that AA accumulation resulting from AA metabolic enzyme inhibition induces apoptosis by regulating gene expression. One primary candidate was PPAR - mediated gene regulation because AA and its metabolites are PPAR ligands and the PPARs are known to be involved in cancer cell apoptosis. We found that AA metabolic inhibitors and exogenous AA do indeed augment PPAR - mediated gene transcription but that this phenomenon is unrelated to the apoptotic induction by these inhibitors. These results agree with our previous findings with regards to 15dPGJ<sub>2</sub>. Using Affymetrix gene array technology we were able to determine that intracellular accumulation of AA and its oxidation to various AA metabolites likely induce apoptosis in neoplastic cells by activating the AP-1 family of stress response transcription factors. These results are included in a manuscript in preparation for submission to *Carcinogenesis*.

## Key Research Accomplishments

- 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> remains the most potent naturally occurring PPAR $\gamma$  agonist identified.
- The degree of PPAR $\gamma$  activation dictates distinct and opposing biological responses in breast cancer cells, ranging from increased proliferation to differentiation and apoptosis.
- 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induced apoptosis requires *de novo* expression of critical gene products.
- Dominant negative expression of PPAR $\gamma$  completely abrogates transcriptional activation induced by 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub>.
- The mechanism of action of 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> is not limited to PPAR $\gamma$  activation. 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> can inhibit NF $\kappa$ B, activate PPAR $\gamma$  and can stimulate reactive oxygen species generation. Together, these events lead to induced expression of key gene products that are involved in PPAR $\gamma$  mediated apoptosis in breast cancer cells.
- 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> is metabolized to polar derivatives by breast cancer cells.
- 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induced cancer cell apoptosis is independent of PPAR $\gamma$ .
- Inhibition of AA metabolism induces intracellular accumulation of AA in neoplastic cells. Apoptosis induced by inhibition of AA metabolic enzymes such as COX is likely due to the accumulation of intracellular AA.
- The AP-1 family of nuclear transcription factors are likely involved neoplastic cell apoptotic signaling in response to AA and its metabolites.

## Reportable Outcomes

### • Manuscripts

1. **Clay CE**, Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH, 2000, 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induces diverse biological responses via PPAR $\gamma$  activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
2. **Clay CE**, Namen AM, Atsumi G, Trimboli AJ, Fonteh AN, High KP, Chilton FH, 2001, The magnitude of PPAR $\gamma$  activation is associated with important and seemingly opposite biological responses in breast cancer cells. *Journal of Investigative Medicine* 49, 413-420
3. **Clay CE**, Atsumi G, High KP, Chilton FH. 2001, 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub>-induced apoptosis requires *de novo* gene expression in breast cancer cells. *Journal of Biological Chemistry* 276, 47131-47135
4. **Clay CE**, Atsumi G, High KP, Chilton FH. Early *de novo* gene expression is required for 15-deoxy-Delta 12,14-prostaglandin J2-induced apoptosis in breast cancer cells. *J Biol Chem*. Dec 2001 14;276(50):47131-5.
5. **Monjazeb AM**, **Clay CE**, High KP, Chilton FH. Antineoplastic properties of arachidonic acid and its metabolites. *Prostaglandins Leukot Essent Fatty Acids*. 2002 Jan;66(1):5-12. Review.
6. **Clay CE**, **Monjazeb AM**, High KP, Chilton FH. 15-Deoxy-delta12,14-prostaglandin J2-induced apoptosis does not require PPAR $\gamma$  in breast cancer cells. *J Lipid Res*. 2002 Nov;43(11):1818-28.
7. **Monjazeb AM**, High KP, Koumenis C, Chilton FH. Inhibitors of Arachidonic Acid Metabolism Act Synergistically to Signal Apoptosis in Cancer Cells. *In preparation*, June 2003
8. **Monjazeb AM**, Connolly AC, Hart LS, High KP, Koumenis C, Chilton FH. Regulation of Gene Expression and Apoptosis by Inhibition of Arachidonic Acid Metabolism. *In preparation*. June 2003

### • Abstracts

1. *PPAR $\gamma$  induced apoptosis requires *de novo* gene expression that is suppressed by a dominant negative mutant in breast cancer cells*. FASEB: Receptors and Signal Transduction, Copper Mountain, CO July 2-9, 2000
2. *15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> inhibits breast cancer cell proliferation via PPAR $\gamma$  activation*. International Society for Preventive Oncology, 5<sup>th</sup> International Meeting, Geneva, Switzerland, October 28-31, 2000, Satellite Symposium October 29, 2000
3. *PPAR $\gamma$  induced apoptosis requires *de novo* gene expression that is suppressed by a dominant negative mutant in breast cancer cells*. Wake Forest University, Breast Cancer Center of Excellence, Winston-Salem, NC, November 16, 2000

4. *PPAR $\gamma$  induced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Keystone Symposium: PPARs a transcription odyssey, Keystone, CO, February 2-9, 2001

- Presentations

1. *PPAR $\gamma$  induced biologic responses require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, Winston-Salem, NC, August 11-12, 2000
2. *15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induced apoptosis is suppressed by a PPAR $\gamma$  dominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000
3. *Mechanisms of Apoptosis in breast cancer cells: 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> and PPAR $\gamma$ .* University of Colorado Health Sciences Center, Denver, CO, February 9, 2001.

- Development of cell lines

1. PPAR $\gamma$  Dominant Negative
2. I $\kappa$ B $\alpha$  Dominant Negative
3. p21 Dominant Negative

- Awards

1. Comprehensive Cancer Center Award: Best graduate student presentation (monetary award) *PPAR $\gamma$  induced biologic changes require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, August 11-12, 2000
2. Avanti Founder's Award: Best graduate student presentation (monetary award and conference expenses) *15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induced apoptosis is suppressed by a PPAR $\gamma$  dominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

- Funding applied for based on work supported by this award

1. Susan G. Komen Breast Cancer Foundation Dissertation Award. *PPAR $\gamma$  Induced Apoptosis Requires de novo Gene Expression in Breast Cancer Cells: searching for key molecular targets.* (submitted March 15, 2001)
2. Wake Forest University Comprehensive Cancer Center. *PPAR $\gamma$  and soy phytoestrogens as possible therapy for breast cancer.* \$10,000 (submitted March 15, 2001)

## **Conclusions**

Naturally occurring derivatives of arachidonic acid metabolism are potent and effective activators of PPAR $\gamma$ . The most potent of these derivatives is 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> (15dPGJ<sub>2</sub>), the dehydration and isomerization product of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>). 15dPGJ<sub>2</sub> induces PPAR $\gamma$  mediated transcriptional activation leading to the synthesis of critical gene products involved in cell cycle arrest and apoptosis. Of these gene products, expression of the cyclin dependent kinase inhibitors, p21 and p27, is associated with marked cell cycle arrest with subsequent apoptosis involving caspase-3. However, apoptosis induced by 15dPGJ<sub>2</sub> is unlikely to be PPAR $\gamma$  mediated as demonstrated by studies with dominant negative forms of this receptor. To further elucidate how AA derivatives such as 15dPGJ<sub>2</sub> induce apoptosis in breast cancer cells investigations into AA metabolic pathways were undertaken. We demonstrate that intracellular accumulation of AA induce apoptosis in cancer cells by activating the AP-1 family of nuclear transcription factors. Given the anti-cancer efficacy of therapies which alter AA metabolism, such as NSAIDs, further investigation into 15dPGJ<sub>2</sub> and other facets of the AA metabolic pathway are warranted.

## References

1. Clay CE, Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH, 2000, 15deoxy $\Delta$ 12,14PGJ<sub>2</sub> induces diverse biological responses via PPAR $\gamma$  activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
2. Clay CE, Namen AM, Atsumi G, Trimboli AJ, Fonteh AN, High KP, Chilton FH, 2001, The magnitude of PPAR $\gamma$  activation is associated with important and seemingly opposite biological responses in breast cancer cells. *Journal of Investigational Medicine* (in press)

## Appendices

Appendix 1: Mechanisms of 15deoxy $\Delta^{12,14}$ PGJ<sub>2</sub> induces apoptosis in breast cancer cells. 15dPGJ<sub>2</sub> induced apoptosis in breast cancer cells requires the expression of critical gene products, such as p21 and p27. However, 15dPGJ<sub>2</sub> also induces the generation of reactive oxygen species which may act on free arachidonic acid (AA) to yield novel nuclear receptor agonists. Moreover, 15dPGJ<sub>2</sub> inhibits key survival signaling protein, such as NF $\kappa$ B and AKT/PKB, and inhibits isopeptidase activity of the ubiquitin proteosome. Together these data show that the extraordinary biological activity of 15dPGJ<sub>2</sub> is a result of PPAR $\gamma$ -dependent and independent mechanisms. Further research is warranted to discern the predominant mechanisms of 15dPGJ<sub>2</sub>-induced apoptosis in breast cancer cells.

